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Amylin-induced central IL-6 production enhances ventromedial hypothalamic leptin signaling

Running title: Amylin-induced IL-6 and hypothalamic leptin signaling

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ABSTRACT

Amylin acts acutely via the area postrema to reduce food intake and body weight but also interacts with leptin over longer periods of time, possibly via the ventromedial hypothalamus (VMH), to increase leptin signaling and phosphorylation of STAT3 (pSTAT3). We postulated that amylin enhances VMH leptin signaling by inducing IL-6 which then interacts with its gp130 receptor to activate STAT3 signaling and gene transcription downstream of the leptin receptor. We found that components of the amylin receptor (RAMPs1-3, CTR1a,b) are expressed in cultured VMH astrocytes, neurons and microglia, as well as in micropunches of arcuate and ventromedial hypothalamic (VMN) nuclei. Amylin exposure for 5 d increased IL-6 mRNA expression in VMH explants and microglia by 2-3 fold as well as protein abundance in culture supernatants by 5- and 2-fold. Amylin had no similar effects in cultured astrocytes or neurons. In rats, 5 d amylin treatment decreased body weight gain and/or food intake and increased ventromedial nucleus (VMN) IL-6 mRNA. Similar 5 d amylin treatment increased VMN leptin-induced pSTAT3 expression in wild type mice and rats infused with lateral ventricular IgG but not in IL-6 knockout mice or rats infused with ventricular IL-6 antibody. Lateral ventricular infusion of IL-6 antibody also prevented the amylin-induced decrease of body weight gain. These results show that amylin-induced VMH microglial IL-6 production is the likely mechanism by which amylin treatment interacts with VMH leptin signaling to increase its effect on weight loss.

INTRODUCTION

Amylin is synthesized by pancreatic β-cells and is co-released with insulin in response to food intake and increasing glucose levels (1). However, while insulin stimulates nutrient disposal and storage, amylin limits nutrient availability by inhibiting food intake, gastric emptying and digestive secretions (2; 3). The amylin receptor is composed of a calcitonin receptor (CTR) heterodimerized with a receptor activity modifying protein (RAMP) (4; 5). There are two splice variants of CTR, 1a and 1b (4; 6) and three known RAMP subtypes (RAMP1, 2, 3) providing six possible combinations for expression of the amylin receptor (7). The CTR can be activated by peptides such as calcitonin and amylin (8). However, CTR has an enhanced affinity for amylin when combined with RAMPs (9). Amylin binds to its receptors which are distributed throughout the brain. These include the area postrema (AP), nucleus of the solitary tract (NTS), the lateral hypothalamic area, ventromedial (VMN) and arcuate (ARC) hypothalamic nuclei and the ventral tegmental area (VTA) (10-14).

Several studies have documented the satiating effect of amylin via its action on the AP (3; 15-17). The VTA has also been demonstrated as a site of amylin's action (18). However, amylin has also been suggested to act in the VMN and ARC to enhance leptin signaling and synergistically decrease food intake and body weight when co-administered with leptin in obese rats and humans, as well as lean rats (19-22). Systemic amylin administration increases expression of the intracellular signaling form of the leptin receptor, Lepr-b, as well as binding of leptin to its receptors in the ARC and VMN. This is associated with an increase in VMN leptin-

induced phosphorylation of signal transducer and activator of transcription-3 (pSTAT3) (19; 20), one of the major signaling pathways downstream of the leptin receptor (23; 24).

Since there is currently no evidence that amylin acting at the AP should increase VMN leptin signaling, we postulated that amylin might act independently in the ventromedial hypothalamus (VMH: ARC + VMN) to stimulate the production of IL-6 which then acts on its receptor signaling complex, IL6 receptor (IL6R) coupled to gp130, to activate STAT3 as a means of increasing downstream leptin signaling. This hypothesis is based on the finding that endogenous IL-6 increases leptin sensitivity (25) and that increased VMH IL-6 production increases leptin signaling and anorectic sensitivity in swim-stressed rats, an effect that is blocked by intraventricular administration of IL-6 antibodies (26). Using *in vivo* and *in vitro* methods, we found that amylin causes VMH microglia to produce IL-6 and increases IL-6 mRNA expression in VMN micropunches from rats treated with amylin. Amylin treatment increased VMN leptin-induced pSTAT3 expression in wild type (wt) mice and rats, but failed to do so in IL-6 knockout (ko) mice or rats infused in their lateral ventricles (LV) with IL-6 antibody. These results strongly suggest that amylin enhances VMH leptin signaling by directly stimulating microglia IL-6 production which then acts on VMH neurons to increase their leptin-induced STAT3 phosphorylation.

RESEARCH DESIGN AND METHODS

Animals

Outbred male Sprague-Dawley rats were purchased from Charles River Laboratories; Wilmington, MA). IL-6 knockout (IL-6 ko; B6;129S6-*Il6*^{tm1Kopf}/J) and wild-type (wt; C57BL/6J) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Rats were housed at 23-24 °C on a reverse 12:12-h light-dark cycle (lights off at 0800) with *ad libitum* access to chow (3.36 kcal/g, 13.5% fat; Purina #5001) and water. Mice were fed mouse chow (3.81 kcal/g, 25% fat; Purina #5015) and housed on a conventional 12:12 light-dark schedule with lights off at 0900. All work was in compliance with the Institutional Animal Care and Use Committee of the E. Orange Veterans Affairs Medical Center.

In Vitro **Amylin Effects**

VMH Explants**:** Postnatal day 21-28 (P21-28) Sprague-Dawley male rats were sacrificed and 350 μm VMH sections (from bregma -2.30 mm to -3.60 mm (27)) were cut with a vibratome in oxygenated slushed aCSF (containing in mmol/L: 118 NaCl, 3 KCl, 1 $MgCl_2$, 2.5 $NaHCO_3$, 1.5 $CaCl^2$, 1.2 NaH_2PO_4, 5 Hepes, 2.5 glucose, 15 sucrose, pH 7.4). Explant slices were transferred to individual wells and maintained in Neurobasal (Invitrogen Grand Island, NY) containing 10% fetal bovine serum (FBS), 5 mmol/L glucose, 10 μg/mL gentamycin, 10000 U/ml Penicillin/Streptomycin at 37 °C for 5 d. They were exposed twice daily to 10 μmol/L amylin (Bachem, Torrance, CA) or PBS control (n=9/group). On day 5, media were collected and stored at -80 °C for cytokine assays. Slices were placed in RNA Later (Ambion, Grand Island, NY), the VMH was punched under microscopic guidance and mRNA expression was assayed by quantitative rt-PCR (QPCR; Applied Biosystems, Grand Island, NY) (28; 29).

Primary VMN neuronal cultures: P21-28 rats were perfused with a 4% sucrose solution and neurons were dissociated from VMN punches as previously described (28; 29). Neurons were cultured in growth media (Neurobasal plus 2.5 mmol/L glucose) for 5 d and exposed twice daily to 10 μmol/L amylin (Bachem, Torrance, CA) or PBS (n=9/group). On day 5, media were collected and kept at -80 °C for cytokine assays. Neurons were exposed to 120 μL of lysis buffer (Ambion, Grand Island, NY) from which mRNA was extracted and gene expression assayed by QPCR (Applied Biosystems, Grand Island, NY) (28).

Primary VMH astrocyte cultures: The VMH was dissected from P21-P28 rats and triturated in Neurobasal-A (Invitrogen, Grand Island, NY) containing 2.5 mmol/L glucose, 0.23 mmol/L sodium pyruvate, 10000 U/mL penicillin/streptomycin, 10 μg/mL gentamycin, 10% FBS at pH 7.4 and astrocytes were dissociated as previously described (30). The day prior to amylin treatment, astrocytes were washed with PBS and serum free Neurobasal-A was added overnight. Astrocytes were then exposed to vehicle alone (PBS) or 10 μmol/L amylin twice daily for 5 d (n=9/group). Terminally, media were collected and stored at -80 °C for cytokine assay. Astrocytes were exposed to 120 μL of lysis buffer (Ambion, Grand Island, NY) followed by mRNA extraction, reverse transcription and quantification by QPCR (Applied Biosystems, Grand Island, NY) (28).

***Primary cortical and hypothalamic microglia cultures*:** Primary mixed glial cortical and hypothalamic cultures were generated from cortical or hypothalamic tissue from P2 rats. Intact brains were removed and dissected free of meninges. Tissue samples were placed in 2% glucose/PBS and digested in 0.25% trypsin for 20 min. Complete Minimum Essential Media

(MEM-C, Invitrogen, Grand Island, NY) containing 10% FBS, glutamine, 10000 U/mL penicillin/streptomycin and 6% glucose were then added. The tissue was gently triturated with a 10 mL pipet and passed through a 130 μm screen. Cells were pelleted at 1200 rpm for 5 min and the pellet was suspended in 10 ml MEM-C and passed through a 35 μm screen. Cells were counted and plated at a density of 1.5 x 10^6 cells/ml. Cells were cultured in 75 cm^2 tissue culture flasks and maintained at 37°C in 5% CO_2. When cultures reached confluency, microglia cells were harvested by shaking at 250 rpm for 90 min, and then pelleted at 1200 rpm for 5 min, suspended in Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12, Invitrogen, Grand Island, NY) containing 10% FBS and plated at a density of 4 x 10^5 cells/ml. At 90% confluency, microglia were treated with vehicle (PBS) or 1 μmol/L amylin twice daily for 5d (n=6/group). Terminally, media were collected and stored at -80°C for cytokine assay. Microglia were treated with 120 μl of lysis buffer (Ambion, Grand Island, NY), mRNA was extracted and assayed by QPCR.

***Tissue Culture Cytokine Measurement*:** Interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor alpha (TNF-α) were measured in culture supernatants using a customized V-Plex proinflammatory assay for rat (K15044D; Meso Scale Discovery [MSD], Gaithersburg, MD). For all assays, culture supernatants were diluted 1 to 2 in the diluent provided. Samples collected from VMH explants and neuronal cultures were incubated in the coated MSD plate for 2 h at room temperature, while samples from astrocyte and microglia cultures were incubated overnight at 4 °C, to increase sensitivity of the assay. Assays were otherwise completed per the manufacturer's instructions. Detection limits of the assay were 6.92 pg/mL for IL-1β, 13.8 pg/mL for IL-6, 16.4 pg/mL for IL-10, and 0.72 pg/mL

for TNF-α. Plates were read using the MESO QuickPlex SQ 120 (MSD) and analyzed using MSD Discovery Workbench analyzer and software package. Cytokine levels were corrected against cyclophilin mRNA to normalize sample size.

***In vivo* Amylin Effects**

After 1 wk of acclimation, 9-10 wk old (300-350 g) rats were randomized by body weight into experimental groups, anesthetized and implanted with subcutaneous, intrascapular minipumps (Alzet Model 2001; Durect Corp., Cupertino, CA) (20)

Experiment 1 (Tables 3, 4, Figure 2): rats were divided into 3 groups: Amylin (dissolved in 0.9% saline infused at 100 μg/kg/d; Bachem, Torrance, CA) treated and fed ad libitum), Pair-fed (0.9% saline infusion with intake matched to previous day's intake by Amylin rats) and Ad-lib fed (0.9% saline infusion). Body weight and food intake were monitored every 2 d. Terminally, food was removed at lights on (2000) the evening before and rats were rapidly decapitated at lights off the next morning. Brains were removed, snap frozen on dry ice and stored at -80 °C for mRNA analysis by quantitative real-time PCR (QPCR) (31). Resulting target gene expression was expressed as a ratio of the constitutively expressed gene, cyclophilin.

Experiment 2 (Figure 3): 9-10 wk old male rats were implanted in the LV (X= 1.6 mm, Y= 0.9 mm, Z= 4 mm -relative to dura) with a cannulae linked to a subcutaneously-implanted osmotic minipump (Azlet Model 2002 and Brain Infusion Kit 2) to infuse either IL-6 antibody (400 ng/kg/day; Santa Cruz sc-7920) or IgG control (400 ng/kg/d Santa Cruz sc-2027). After 5 d recovery, rats were implanted subcutaneously with a second osmotic minipump (Alzet 2001) for

saline (0.9%) or amylin (100 μg/kg/d) for an additional 5 d. Body weight and food intake were monitored daily. Terminally, food was removed 2 h before lights off (0700). Rats were injected with murine leptin (5 mg/kg, i.p. in PBS; NHPP, Torrance, CA) at lights off, anesthetized (ketamine/xylazine) 45 min later and rapidly perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in PBS. Brains were removed, post-fixed overnight in 4% PFA and transferred the next day to KPBS containing 20% sucrose for 24h. The brains were then frozen in 2-methylbutane.

Amylin Effects on IL-6 Knockout (IL-6 ko) Mice

9 wk old IL-6 ko and wild type (wt) mice were fed mouse chow *ad libitum* throughout the experiment. After 1 wk of acclimation, mice were randomized by body weight into four groups, anesthetized and implanted with subcutaneous, intrascapular minipumps (Alzet Model 1002; Durect Corp., Cupertino, CA). Pumps contained amylin (Bachem, Torrance, CA) in 0.9% saline (wt-A and IL-6 ko-A infused at 1 mg/kg/d) or 0.9% saline vehicle (wt-S and IL-6 ko-S). Body weight and food intake were monitored bi-weekly for 2 wk. Terminally, food was removed at 2 h before lights off. Mice were injected with murine leptin (5 mg/kg, i.p. in PBS; NHPP, Torrance, CA) at lights off, anesthetized (ketamine/xylazine) 45 min later and rapidly perfused with 0.9% saline followed by 2% PFA in PBS (32). Brains were removed, post-fixed overnight in KPBS containing 20% sucrose and frozen with 2-methylbutane.

^{125}I Amylin Receptor Binding Autoradiography: Brains from non-fasted rats were removed, frozen on powdered dry ice and 12 μm sections were cut through the midpoint of the ARC, VMN and dorsomedial nucleus (DMN) pars compacta (27), mounted on gel-coated slides,

desiccated and stored at -80 °C. Amylin receptor binding was carried out by methods adapted from Sexton *et al*. (33). Briefly, sections were thawed and rinsed in incubation buffer (20 mmol/L HEPES containing 100 mmol/L NaCl, 1 mg/mL BSA and 0.5 mg/mL Bacitracin). Sections were then incubated at room temperature for 1 h in incubation buffer containing 70-75 pmol/L ^{125}I amylin (NEX44; Perkin Elmer, Boston, MA) plus 1 μmol/L unlabeled rat amylin (non-specific "binding"; Bachem, Torrance, CA). Slides were rinsed in incubation buffer at 4 °C and rinsed two more times at 4 °C in modified incubation buffer (20 mmol/L HEPES containing 100 mmol/L NaCl). After a brief dip in dH_2O, sections were dried under forced cold air and desiccated for 24 h. Sections were then exposed to BioMax MR Film (Kodak, Rochester, NY) at -80 °C for 7-14 d.

pSTAT3 Immunohistochemistry: Brain sections (30 μm) were cut through the mid-VMH (27) from saline and amylin-treated wt and IL-6ko mice and mounted on Superfrost Plus slides. Free-floating (30 μm) sections were cut from brains of control and amylin-treated rats infused in the LV with IgG or IL-6 antibody. Slides and free-float sections were stored in antifreeze at -20 °C until processed for leptin-induced pSTAT3 immunohistochemistry using rabbit anti-pSTAT3 antibody (1:1000; Cell Signaling Tech., Danvers, MA) using methods previously described (31). Three consecutive sections were counted per brain using a Bioquant image analysis system (Bioquant, Nashville, TN) by an experimentally naive observer.

Statistics: Statistical comparisons among variables for *in vivo* studies were made by 1-way ANOVA with Bonferroni post-hoc analysis. Body weight gain and food intake were analyzed by 1-way ANOVA with repeated measures. Food efficiency was calculated by dividing the body

weight gain in grams by the total food intake in kcal and multiplying the total by 1000. All data are expressed as mean ± SEM. Statistical analysis was performed using SYSTAT software (SYSTAT, Chicago, IL). Comparisons between control and amylin-treated groups in *in vitro* studies were assessed using t-test for nonparametric statistics (GraphPad Prism, La Jolla, CA).

RESULTS

Distribution of ^{125}I Amylin Binding

We confirmed previous findings (33; 34) that ^{125}I amylin binds to the ventromedial portion of the VMN, as well as the ARC, dorsomedial nucleus, perifornical and medial tuberal hypothalamus and the medial amygdalar nucleus (Fig. 1). There was little binding in cerebral cortex or hippocampal structures at the rostro-caudal level through the midpoint of the VMH. Hindbrain structures were not examined since the emphasis here was on the effects of amylin on forebrain structures. No amylin binding was found in sections co-incubated with unlabeled amylin (See supplemental Figure 1).

In Vitro effects of Amylin on Hypothalamic Explants, Neurons, Astrocytes and Microglia.

Exposing VMH explants to 10 μmol/L amylin for 5 d increased IL-6 mRNA expression by 320% (Table 1) and secretion of IL-6 protein 5.5-fold (Table 2). Amylin also increased mRNA expression of RAMP1 and 2 subunits of the amylin receptor by 122% and 103%, respectively, while it decreased expression of the CTR1b subunit of the amylin receptor by 72% (Table 1). In addition, amylin increased IL-10 secretion 7-fold (Table 2).

To assess the specific cellular source of IL-6 production within the VMH, primary cultures of VMH neurons, microglia and astrocytes, as well as cerebral cortical microglia were incubated with amylin (1-10 μmol/L) for 5 d. Exposure of primary hypothalamic microglial cultures from P2 rats to 1 μmol/L amylin increased IL-6 mRNA expression by 211% (Table 1) and IL-6 protein production by 204% (Table 2). Amylin also increased microglial CTR1b mRNA expression by 56%, and decreased both leukemia inhibitory factor (LIF), a member of the IL-6-type class cytokine family that acts though gp130, and gp130 mRNA expression by 29% (Table 1). The amylin-induced increase in IL-6 mRNA expression was not specific to hypothalamic microglia since amylin also increased cerebral cortex microglial IL-6 mRNA expression by 140% (Table 1) and IL-6 media secretion by 310% (Table 2). Amylin also increased the secretion of TNF-α by cortical microglia by 158% (Table 2). Amylin exposure had no effect on neuronal cytokine mRNA or protein production (Tables 1 and 2), although it did increase neuronal SOCS3 (an inhibitor of JAK/STAT3 signaling) mRNA expression by 33% (Table 1). Similarly, while amylin had no effect on cultured astrocyte IL-6 mRNA expression, it did increase TNF-α mRNA by 113%, IL-1β by 211% and ciliary neurotrophic factor (CNTF) by 74%, while decreasing LIF expression by 61% (Table 1).

***In Vivo* Effects of Amylin on VMH Cytokine Production (Experiment 1)**

Male, 9-10 wk old rats were infused subcutaneously with either amylin or vehicle for 5 d. Rats pair-fed to amylin-treated rats served as additional controls. Amylin-treated rats consumed 24% fewer kcals overall ($p=0.001$, Table 3; Fig. 2B) and gained 86% less body weight compared to ad libitum-fed controls over 5 d of treatment (Table 3; Fig. 2A). This resulted in an 82% lower feed-efficiency overall in amylin-treated rats suggesting an amylin-induced increase in energy

expenditure (Table 3). In VMN micropunches from these rats, expression of IL-6 mRNA was increased by 46% in amylin-treated rats vs. ad libitum controls, while pair-feeding had no effect on IL-6 expression (Table 4). Associated with the increase in VMN IL-6 expression, VMN Lepr-b mRNA expression was increased by 60% (Table 4) compared to pair-fed controls. Also, expression of VMN CTR1a and b were increased by 120% and 176%, respectively, compared to pair-fed rats (Table 4). The amylin-induced changes appeared to be specific to IL-6 as amylin had no effects on the mRNA expression of any other VMN or ARC cytokine. Despite the lack of significant amylin-induced changes in IL-6 or Lepr-b expression in the ARC, amylin-treated rats had significant increases in both NPY and AgRP mRNA expression compared to ad libitum or pair-fed controls (Table 4).

Amylin Effects on Rat VMH Leptin Signaling of LV IL-6 Antibody Infusions (Experiment 2)

To confirm the hypothesis that the amylin sensitizing effect on leptin signaling is due to an amylin-induced increase in IL-6 activation of JAK/STAT3 signaling, IgG or IL-6 antibodies were infused into the LV of rats for 5 d. At that time, rats were then additionally infused subcutaneously with either amylin or vehicle for 5 d more. Neither IgG nor IL-6 antibodies altered food intake or body weight gain over the first 5 d of LV infusions (Fig. 3A, B). After an additional 5 d of amylin treatment, LV IgG-infused rats decreased their body weight gain and food intake by 96% and 27%, respectively, as compared to IgG-Saline rats (Fig. 3C, D). On the other hand, LV IL-6 antibody infusion attenuated the amylin-induced decrease in body weight gain by 37% (Fig. 3C) but had no effect on amylin-induced reduction in food intake (Fig. 3D). Most importantly, 10 d of IL-6 antibody treatment and 5 d of amylin infusion prevented the

amylin-induced enhancement of leptin-induced VMN pSTAT3 expression seen in IgG Amylin rats by 25% (Fig.3E). However, IL-6 antibody infusion had no effect on amylin's enhancement of leptin-induced pSTAT3 expression in the ARC (Fig.3E). These data strongly suggest that IL-6 is required for the amylin sensitizing effects on VMH leptin signaling through which it contributes to amylin-induced reductions in body weight gain but not food intake.

Effects of Amylin on Leptin Signaling in IL-6 ko Mice

To further confirm the hypothesis that the amylin sensitizing effect on leptin signaling is due to an amylin-induced increase in IL-6 activation of JAK/STAT3 signaling, wt and IL-6 ko mice were infused with either amylin or vehicle by minipumps for 2 wk. Although there were no significant differences in body weight gain or food intake among the groups, there was a trend towards decreased body weight gain in amylin-treated wt controls (~~Fig. 4A, B~~ See supplemental Figure 2). Most importantly, 2 wk amylin treatment was associated with a 67% increase in leptin-induced pSTAT3 expression selectively in the VMN of wt but not IL-6 ko mice (Fig. 4~~C, D~~ A, B). These data strongly suggest that IL-6 is required for the amylin sensitizing effects of VMH leptin signaling.

DISCUSSION

The goal of this study was to identify the mechanism by which systemic amylin administration increases VMH leptin signaling to produce a synergistic effect on weight loss in obese individuals (19; 20). We first confirmed that ^{125}I amylin binds in the VMH (as well as other forebrain areas) and demonstrated, for the first time, that CTR1a and b along with RAMP1-3

components of the amylin receptor complex are variably expressed in VMH microglia, astrocytes and neurons. We postulated that amylin interacts with leptin signaling by causing cells within the VMH to produce IL-6 which is known to phosphorylate STAT3, a common downstream mediator of leptin signaling (35; 36), via its IL6R complex. We found that amylin did, indeed, increase IL-6 production in both VMH explants and VMN micropunches from rats treated in vivo with amylin and that this occurred selectively in microglia but not astrocytes or neurons. As previously demonstrated (19; 20), amylin reduced body weight gain and food intake in adult rats; this reduced body weight gain was partially reversed by LV infusions of IL-6 antibody. While high doses of amylin failed to reduce body weight gain or food intake in wt or IL-6 ko mice, amylin's enhancement of VMN leptin-induced pSTAT3 expression was completely inhibited in IL-6 ko mice and rats with LV IL-6 antibody infusions. The failure of LV IL-6 antibody infusions in rats to completely prevent amylin-induced reductions in body weight gain or food intake was not unexpected since amylin is known to produce weight loss and anorexia via its actions in the AP (37; 38), as well as in the VTA (18). The failure of LV IL-6 antibody to reverse the amylin-induced reduction in food intake suggests that the primary role of amylin-induced enhancement of VMH leptin signaling via microglial IL-6 production is in affecting energy expenditure. This also suggests that amylin's effects on reducing food intake are not mediated via its actions on VMH leptin signaling, but rather via its actions on other brain sites; however, it is also possible that the dose of IL-6 antibody used was not sufficient to prevent the effect of amylin on food intake. Therefore, because the partial blockade of amylin-induced body weight reduction by LV IL-6 antibody infusion was not paired with a decrease in food intake, this suggests that amylin's enhancement of leptin signaling in the VMH likely resulted in increased energy expenditure.

Our data strongly support the hypothesis that amylin's enhancement of VMH leptin signaling (20) is due to its direct action on VMH microglia to produce IL-6 which then acts on its IL6R/gp130 receptor complex (39) to activate STAT3 phosphorylation which is also downstream of Lepr-b signaling (40). Activation of STAT3 results in its dimerization and translocation into the nucleus where it then alters gene transcription (24). Given the fact that systemic amylin treatment increases VMH Lepr-b gene expression, binding of leptin to its cell surface receptor in both the ARC and VMN and leptin-induced pSTAT3 expression in the VMN (19; 20), our data support the hypothesis that convergence of amylin-induced microglial production of IL-6 on STAT3 activation is an important route by which amylin enhances leptin signaling in the VMH. Here we confirmed the previous finding that systemic amylin treatment increases leptin-induced pSTAT3 expression in the VMN of wt mice (20) and show, for the first time, that amylin had no effect on VMN pSTAT3 expression in IL-6 ko mice. Amylin's enhancement of VMN leptin-induced pSTAT3 expression was also inhibited by LV IL-6 antibody infusion in rats. Such a direct action in the VMH requires that amylin cross the blood-brain barrier which has been shown to occur (41; 42). However, since amylin exposure did not increase Lepr-b expression in isolated VMH explants as it does when given *in vivo* (20), there may be an additional component of amylin's effect on leptin signaling that is required to alter Lepr-b expression in the intact animal.

Interestingly, amylin exposure had differential effects on CTR1 and RAMP expression. For example, amylin exposure increased RAMP1 and 2 in VMH explants and RAMP2 and 3 in cortical, but not VMH microglial cultures. However, systemic amylin administration *in vivo* had no consistent effect on ARC or VMN RAMP expression. Similarly, CTR1b expression was

differentially altered depending upon the tissue examined and type of exposure. These results suggest that there are clear differences between the responses of cortical and VMH microglia to amylin, just as cortical and hypothalamic astrocytes differ in their characteristics (43). Given the fact that amylin affected CTR and RAMP expression only in cultured microglia but not astrocytes or neurons, these results demonstrate feedback by amylin on its own receptor selectively in microglia. Similarly, while amylin stimulated VMH microglial IL-6 expression, it also had a negative feedback effect (44) on the expression of the gp130 component of the IL-6 receptor complex (45). In fact, the gp130 family of receptors can be activated by other cytokines such as LIF (39; 46), although in our case, amylin altered LIF expression only in cultured hypothalamic astrocytes and this was an inhibitory rather than a stimulatory effect.

Although numerous studies (2; 19; 20; 47-49), including ours here in rats, have clearly shown that amylin acts alone to decrease food intake and body weight in obese and lean rats and obese humans, it had no such effects in wt mice treated for 2 wk with amylin doses that were 10 times higher than those used in rats, despite showing a clear enhancement of wt leptin signaling in the VMN. There is no ready explanation for this lack of effect on body weight or food intake in amylin-treated wt mice. It is possible that amylin treatment reduced their adiposity but this could not be assessed due to methodological requirements for later immunohistochemistry. Also, although 5 d of pair-feeding to amylin-treated rats had no effect on ARC NPY or AgRP expression, amylin treatment actually increased the expression of these orexigenic peptides. Since all of the changes in amylin-induced IL-6 production and leptin signaling occurred selectively in the VMN, to the exclusion of ARC Lepr-b-expressing neurons, it is possible that

the upregulation of these peptides was indirectly mediated by alterations in VMN leptin signaling.

In conclusion, we have demonstrated that, in addition to amylin's well-known direct effect on AP and VTA neurons which mediates much of its anorectic effects (3; 15-18), amylin also acts directly to stimulate VMH microglia production of IL-6. This IL-6 is released into the interstitial space where it acts on its IL-6/gp130 receptor on Lepr-b-expressing neurons in the VMN to enhance leptin's activation of STAT3 phosphorylation. While amylin acts directly in the AP to decrease food intake and body weight, especially acutely (15; 47; 50), its interaction with leptin on weight loss in obese rats and humans appears to depend on its ability to stimulate VMN microglial IL-6 production to increase leptin signaling (2; 19; 47; 48). This novel discovery provides a potential avenue for discovery of new leptin sensitizers in the treatment of obesity.

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Table 1: Amylin-induced changes in VMH explant, neuron, astrocyte, hypothalamic and cerebral cortex microglia gene expression

	Explant		Neurons		Astrocytes		Hypothalamic Microglia		Cortical Microglia	
	Control	Amylin	Control	Amylin	Control	Amylin	Control	Amylin	Control	Amylin
IL 6	0.77±0.35	3.24±0.87*	1.39±0.21	1.89±0.17	1.01±0.11	0.78±0.07	0.53±0.07	1.65±0.39*	0.68±0.10	1.63±0.25*
Il 1-β	1.30±0.29	1.71±0.20	1.17±0.20	1.23±0.19	0.47±0.05	1.46±0.16*	0.76±0.09	1.32±0.22	0.94±0.14	1.21±0.12
IL-10	Und.	Und.	Und.	Und.	Und.	Und.	0.89±0.13	0.97±0.19	0.57±0.11	1.11±0.14*
TNF-α	1.20±0.30	1.86±0.40	1.24±0.20	1.40±0.21	0.67±0.12	1.43±0.17*	2.16±0.28	1.66±0.31	1.07±0.10	0.95±0.12
LIF	0.78±0.28	0.41±0.15	1.01±0.11	1.08±0.11	1.40±0.10	0.55±0.06*	1.07±0.07	0.76±0.07*	0.90±0.06	1.08±0.08
CNTF	0.88±0.19	0.69±0.16	1.34±0.26	1.50±0.34	0.74±0.08	1.29±0.16*	1.16±0.08	0.93±0.05	0.95±0.07	0.95±0.12
gp130	0.17±0.04	0.21±0.10	1.44±0.27	1.20±0.18	1.02±0.05	0.95±0.02	1.21±0.13	0.86±0.07*	1.00±0.07	1.16±0.20
CTR1a	2.41±0.54	3.80±1.35	1.26±0.05	1.30±0.03	0.71±0.17	0.65±0.1	0.87±0.12	1.13±0.18	0.78±0.12	1.35±0.21
CTR1b	5.92±0.53	1.65±0.63*	1.03±0.04	1.01±0.06	Und.	Und.	0.75±0.11	1.17±0.10*	Und	Und
RAMP1	1.23±0.32	2.74±0.49*	0.94±0.05	0.96±0.09	0.78±0.06	1.03±0.12	1.14±0.10	0.87±0.12	1.07±0.09	0.92±0.08
RAMP2	0.29±0.04	0.59±0.10*	1.03±0.06	1.01±0.07	0.93±0.08	0.97±0.1	1.07±0.09	1.02±0.05	0.95±0.04	1.10±0.04*
RAMP3	0.89±0.27	1.15±0.15	1.00±0.04	0.96±0.04	0.99±0.07	1.13±0.06	0.74±0.15	1.07±0.13	0.80±0.05	1.24±0.05*
Lepr-b	1.89±0.60	1.73±0.70	0.75±0.06	0.55±0.06						
SOCS3	0.59±0.20	0.47±0.15	0.98±0.07	1.30±0.12*						

VMH explants, neurons, astrocytes and microglia were incubated with amylin (1-10μmol/L) vs. vehicle (PBS) for 5 days: Data are mean ± SEM of duplicate determinations expressed relative to the amount of the mRNA expression of cyclophilin (n=9/group). *P<0.05 or less control vs. amylin in each type of cell culture. Und=Undetectable.

Table 2: Cytokine production after 5d treatment of amylin (1-10μmol/L) in VMH explant, VMH neurons, VMH astrocytes and cortex and hypothalamic microglia from male SD rats.

	Explant		Neurons		Astrocytes		Hypothalamic microglia		Cortical microglia	
	Control	**Amylin**	**Control**	**Amylin**	**Control**	**Amylin**	**Control**	**Amylin**	**Control**	**Amylin**
IL-6	368±83	2058±241*	64.4±6.6	82.1±7.2	15.1±1.82	11.3±1.50	28.6±6.59	86.9±21.6*	18.6±3.92	76.5±10.21*
IL-10	2.46±0.95	18.1±4.62*	2.21±0.41	3.02±0.61	3.75±0.72	3.95±0.57				
IL-1β			7.22±1.17	8.27±2.40	11.9±0.75	10.8±1.52	9.19±2.23	12.54±2.81	11.24±3.54	15.3±1.80
TNFα	5.27±1.51	12.4±3.32	8.83±0.56	9.88±0.96	0.47±0.05	0.56±0.05	2.44±0.56	3.15±0.72	1.48±0.44	3.82±0.55*

Cytokine levels in the supernatant of cultures in pg/mL were normalized to the amount of cyclophilin mRNA expression in each tissue, respectively. N=9-6/group *P<0.05 or less by t-test vehicle vs. amylin.

Table 3: Effects of 5d of systemic amylin (100µg/kg/d) vs. vehicle (0.9% saline) infusions in rats

	Ad-Lib Fed	Amylin	Pair-Fed
Body Weight Initial, g	346 ± 2.9	346 ± 2.6	346 ± 3.8
Final Body Weight, g	369 ± 3.6[a]	349 ± 3.2[b]	356 ± 3.2[b]
5d Body Weight Gain, g	22 ± 3.8[a]	3.1 ± 2.5[b]	9.6 ± 1.9[ab]
5d Food Intake, kcal	384 ± 8.6[a]	290 ± 8.3[b]	290 ± 7.5[b]
5d Feed Efficiency ((body weight gain(g)/ food intake (kcal))*1000)	51± 10[a]	9 ± 9[b]	32 ± 6[ab]

Values are mean ± SEM. N=9-10/group. Parameters with differing superscripts differ from each other by P<0.05 or less.

Table 4: ARC and VMN gene expression after 5d of systemic amylin (100µg/kg/d) vs. vehicle (0.9% saline) infusion in rats

	ARC			VMN		
	Ad-Lib Fed	**Amylin**	**Pair-Fed**	**Ad-Lib Fed**	**Amylin**	**Pair-Fed**
IL-6	1.29 ± 0.20	1.44 ± 0.08	1.25 ± 0.17	1.66 ± 0.21[a]	2.43 ± 0.15[b]	1.81 ± 0.21[ab]
IL-1β	0.81 ± 0.18	0.74 ± 0.12	0.80 ± 0.15	1.54 ± 0.27	1.06 ± 0.17	1.27 ± 0.24
TNFα	1.96 ± 0.18	1.60 ± 0.21	1.64 ± 0.16	1.28 ± 0.18	1.10 ± 0.14	1.15 ± 0.11
LIF	1.24 ± 0.13	1.05 ± 0.11	1.21 ± 0.08	0.91 ± 0.05	0.89 ± 0.03	1.04 ± 0.06
CNTF	1.56 ± 0.13	1.52 ± 0.10	1.82 ± 0.13	1.16 ± 0.07	1.25 ± 0.04	1.15 ± 0.05
gp130	2.53 ± 0.21	2.35 ± 0.15	2.36 ± 0.15	1.40 ± 0.07	1.49 ± 0.06	1.27 ± 0.06
Lepr-b	0.81 ± 0.06	0.83 ± 0.07	0.82 ± 0.08	0.93 ± 0.11[ab]	1.20 ± 0.10[a]	0.75 ± 0.06[b]
SOCS3	1.33 ± 0.14	1.17 ± 0.06	1.42 ± 0.10	1.15 ± 0.16	1.05 ± 0.13	0.87 ± 0.11
RAMP1	0.71 ± 0.07	0.74 ± 0.05	0.99 ± 0.10	1.14 ± 0.08	1.05 ± 0.07	1.20 ± 0.08
RAMP2	1.22 ± 0.08	1.23 ± 0.06	1.23 ± 0.08	1.09 ± 0.04[a]	1.04 ± 0.04[ab]	0.95 ± 0.03[b]
RAMP3	0.87 ± 0.04	0.90 ± 0.07	1.04 ± 0.11	0.89 ± 0.10	1.08 ± 0.11	0.75 ± 0.09
CTR1a	1.17 ± 0.10	1.23 ± 0.07	1.14 ± 0.07	0.72 ± 0.14[ab]	1.01 ± 0.15[a]	0.46 ± 0.10[b]
CTR1b	1.06 ± 0.09	1.17 ± 0.07	0.97 ± 0.08	0.89 ± 0.15[ab]	1.27 ± 0.18[a]	0.46 ± 0.08[b]
InsR	1.10 ± 0.07	1.03 ± 0.04	1.07 ± 0.05	0.94 ± 0.06	1.06 ± 0.05	0.91 ± 0.05
NPY	0.84 ± 0.12[a]	1.33 ± 0.10[b]	0.96 ± 0.07[a]			
AgRP	0.84 ± 0.10[a]	1.20 ± 0.07[b]	0.80 ± 0.04[a]			
POMC	1.12 ± 0.11	1.10 ± 0.12	1.06 ± 0.10			

Values are mean ± SEM of duplicate determined mRNA levels as compared to relative mRNA levels of cyclophilin. N=9-10/group. Parameters with differing superscripts differ from each other by P<0.05 or less.

FIGURE LEGENDS

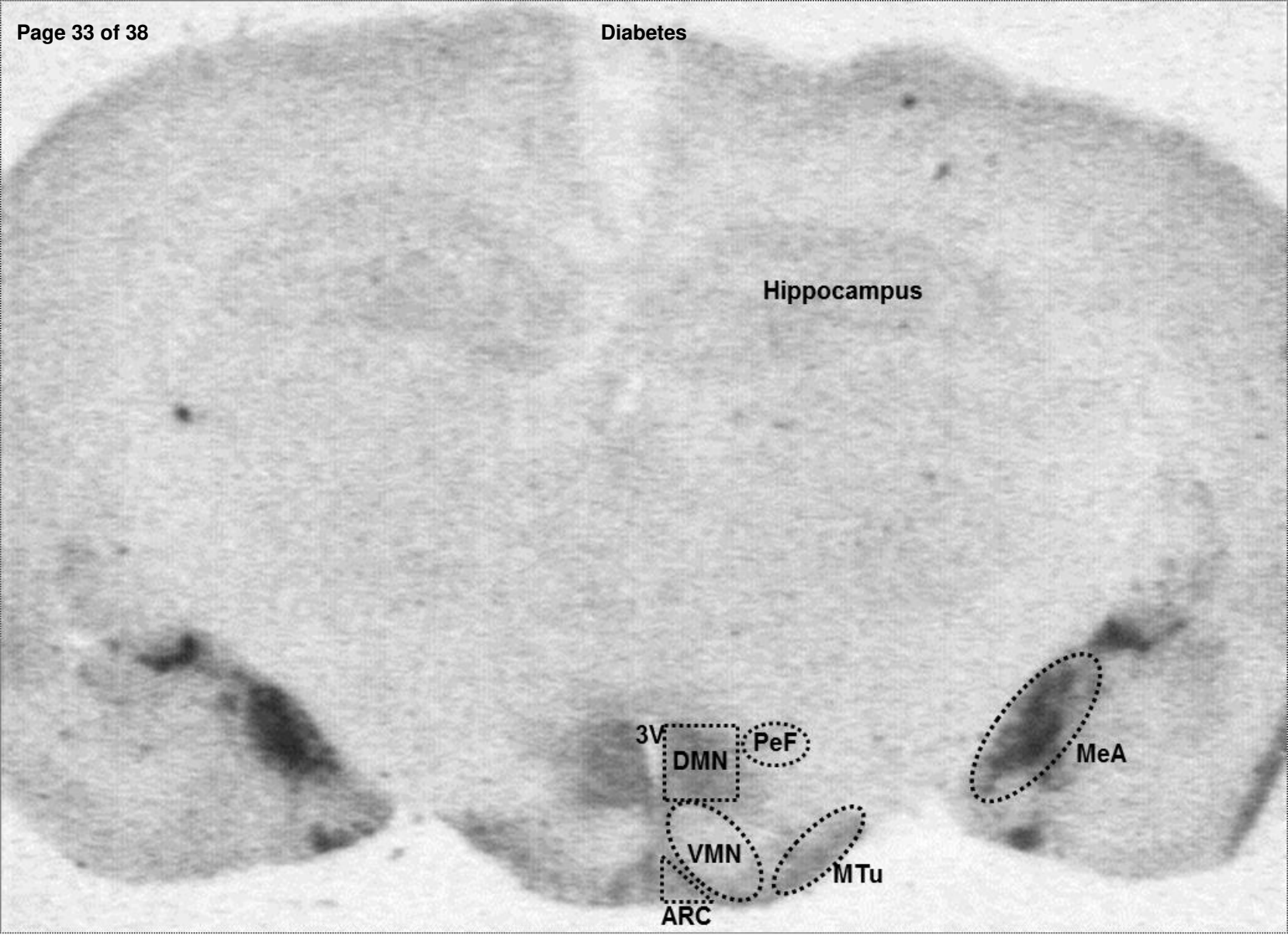
Figure 1: ^{125}I amylin binding in chow-fed rats. *Dotted areas*, hypothalamic ARC (arcuate), VMN (ventromedial nucleus), DMN (dorsomedial nucleus), Perifornical (PeF) and MTu (Medial Tuberal nucleus), MeA (Medial Amygdala) identified on the cresyl violet-stained slides used to generate the autoradiogram.

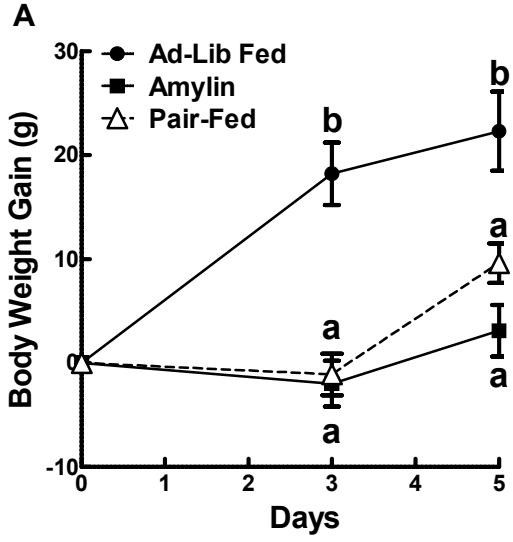
Figure 2 (Experiment 1): Body weight gain (**A**) and food intake (**B**) in ad-libitum (Ad-Lib), amylin and pair-fed 9-10 wk old male rats after 5 d of systemic amylin (100µg/kg/d) vs. vehicle (0.9% saline) infusion with osmotic minipump. Vehicle was infused in Ad-Lib Fed and Pair-Fed groups. Values are mean ± SEM. N=9-10/group *P<0.05 or less.

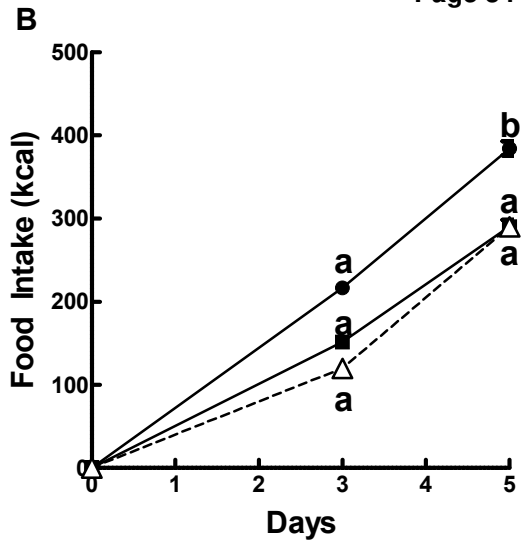
Figure 3 (Experiment 2): Body weight gain (**A, C**) and food intake (**B, D**) of LV infusions of IgG vs. IL-6 antibody for 5 d (**A**, **B**) followed by 5 d of systemic amylin (100µg/kg/d) vs. vehicle (0.9% saline) with osmotic minipump (**C**, **D**) in 9-10 wk old rats. Effect of LV infusions of IgG vs. IL-6 antibody and systemic amylin vs. saline on leptin-induced (5 mg/kg, ip) pSTAT3 immunohistochemistry in the ARC and VMN (**E**). Images taken at 10X magnification (**F**, **G, H**). Values are mean ± SEM. N=8/group. Parameters with differing superscripts differ from each other by P<0.05 or less.

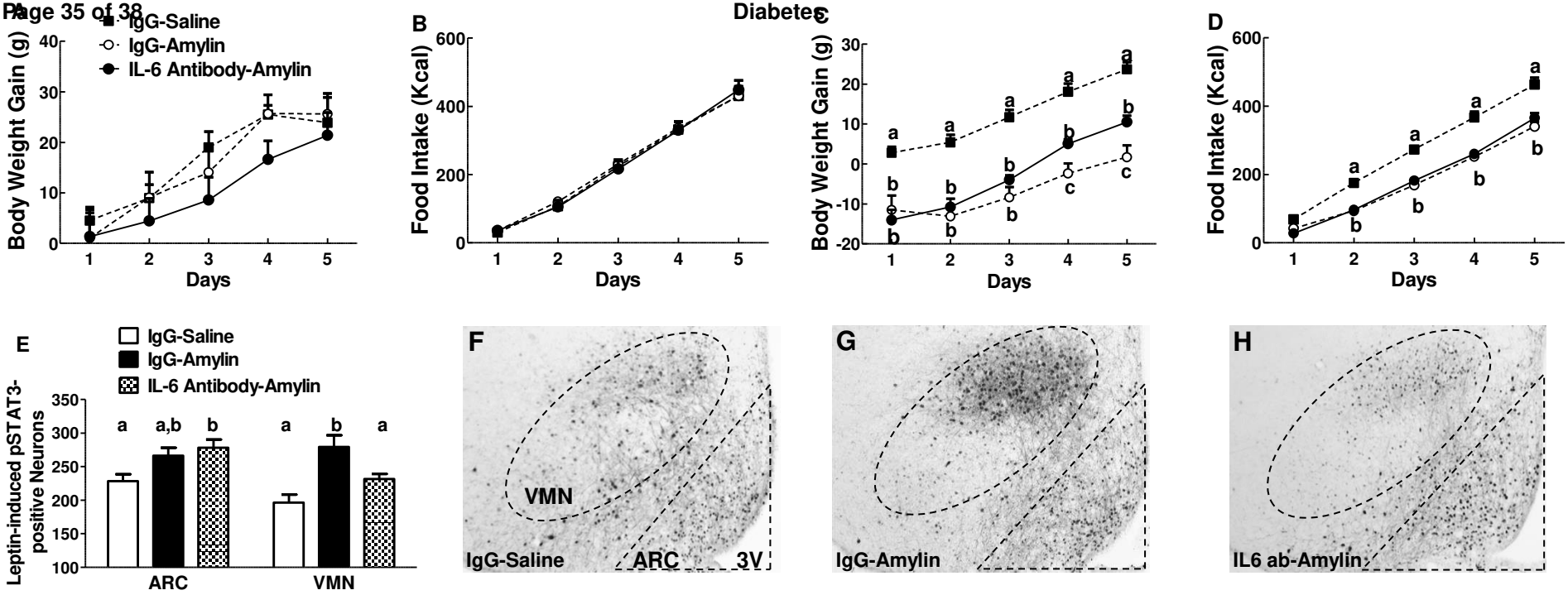
Figure 4: Effect of systemic amylin or saline on leptin-induced (5 mg/kg, ip) pSTAT3 immunohistochemistry in the ARC (**A**) and VMN (**B**) of saline- (wt-S) vs. amylin-treated (A; 1mg/kg/day) wild type (wt-A) and saline- (IL-6 ko-S) vs. amylin-treated IL-6 knockout (IL-6 ko-A) mice after 2 wk of systemic amylin (1mg/kg/d) vs. vehicle (0.9% saline) infusion with

osmotic minipump. Images taken at 20X magnification of wt-S (**C**) vs. IL-6 ko-S (**E**) and wt-A (**D**) vs. IL-6 ko-A (**F**). Values are mean ± SEM. N=8/group *P<0.05 or less wt-S vs. wt-A mice.

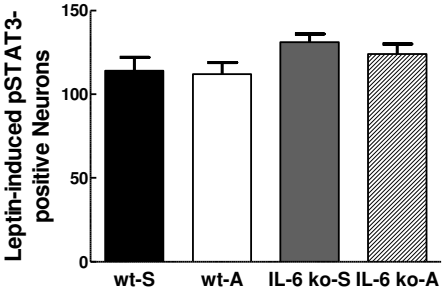

Hippocampus
3V
DMN
PeF
MeA
VMN
MTu
ARC


A
Ad-Lib Fed
Amylin
Pair-Fed
Body Weight Gain (g)
30
20
10
0
-10
0
1
2
3
4
5
Days
b
b
a
a
a
a

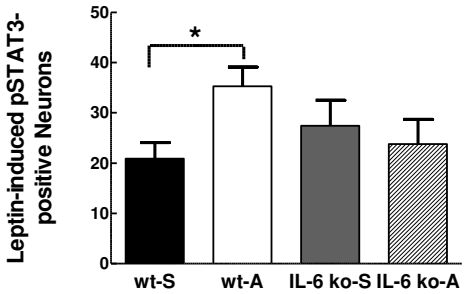

B
Food Intake (kcal)
500
400
300
200
100
0
0
1
2
3
4
5
Days
b
a
a
a
a
a

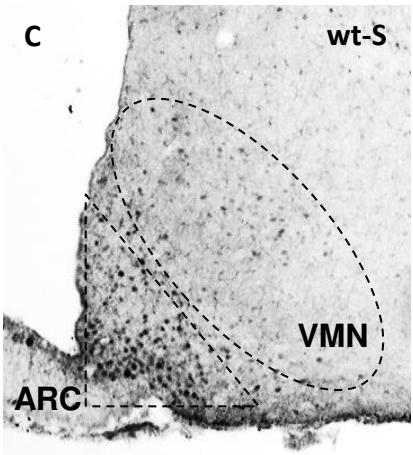

A
IgG-Saline
IgG-Amylin
IL-6 Antibody-Amylin
Body Weight Gain (g)
Days
B
Food Intake (Kcal)
Days
C
Body Weight Gain (g)
Days
D
Food Intake (Kcal)
Days
E
IgG-Saline
IgG-Amylin
IL-6 Antibody-Amylin
Leptin-induced pSTAT3-positive Neurons
ARC
VMN
F
VMN
IgG-Saline
ARC
3V
G
IgG-Amylin
H
IL6 ab-Amylin

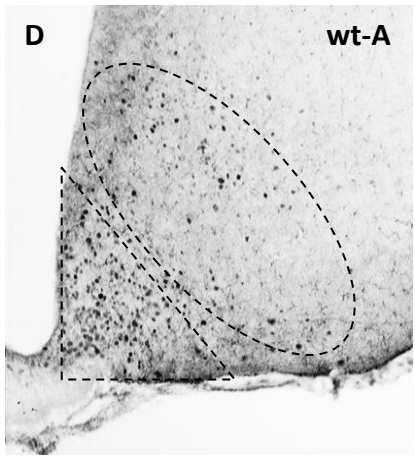
A

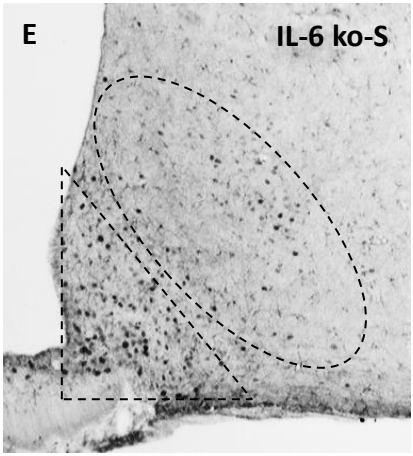

Leptin-induced pSTAT3-positive Neurons
150
100
50
0
wt-S
wt-A
IL-6 ko-S
IL-6 ko-A

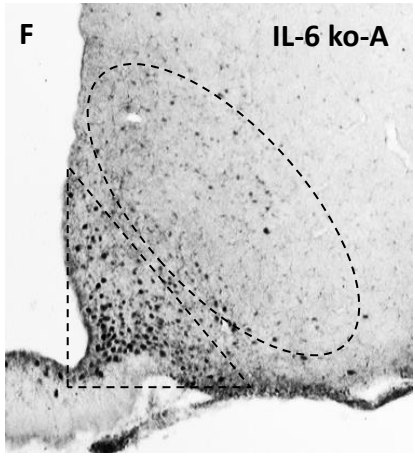
B


Leptin-induced pSTAT3-positive Neurons
50
40
30
20
10
0
*
wt-S
wt-A
IL-6 ko-S
IL-6 ko-A


C
wt-S
VMN
ARC

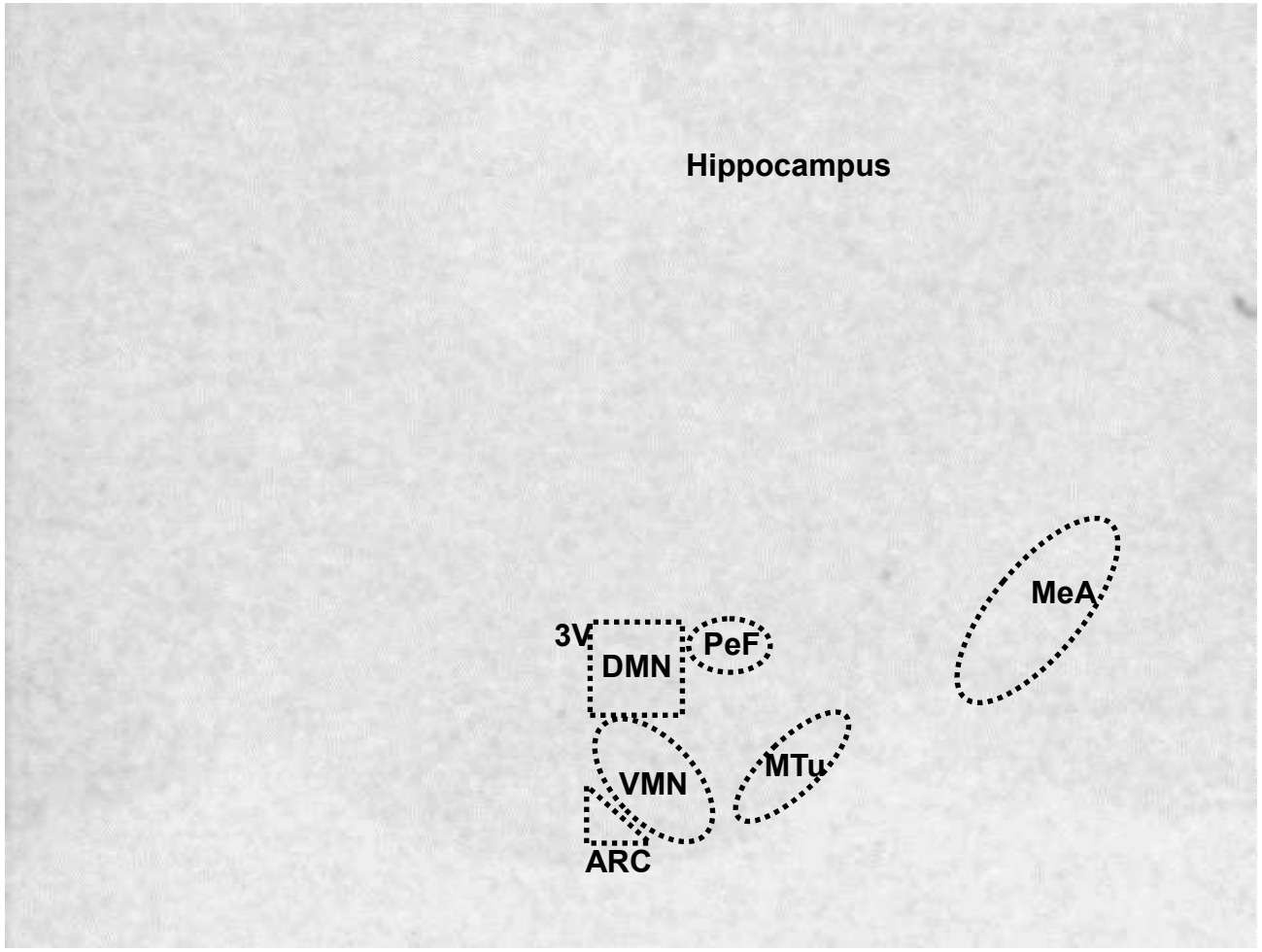

D
wt-A


E
IL-6 ko-S


F
IL-6 ko-A

Supplementary figure 1

Unlabelled amylin binding in chow-fed rats. *Dotted areas*, ARC (arcuate), VMN (ventromedial nucleus), DMN (dorsomedial nucleus), Perifornical (PeF), MTu (Medial Tuberal nucleus), MeA (Medial Amygdala) identified on the cresyl violet-stained slides used to generate the autoradiogram.



Supplementary figure 2

Body weight gain (**A**) and Food intake (**B**) of saline- (wt-S) vs. amylin-treated (A; 1mg/kg/day) wild type (wt-A) and saline- (IL-6 ko-S) vs. amylin-treated IL-6 knockout (IL-6 ko-A) mice after 2 wk of systemic amylin (1mg/kg/d) vs. vehicle (0.9% saline) infusion with osmotic minipump. Values are mean ± SEM. N=8/group *P<0.05 or less wt-S vs. wt-A mice. NB: all 4 groups are represented in B but overlap almost completely.